A Molecular Study of Hemotropic Mycoplasmas (Hemoplasmas) In Cats in Iran

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Running header: Hemoplasma molecular study

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Abstract:

Background; Three feline hemoplasma species are recognized; *Mycoplasma haemofelis*, *Candidatus* Mycoplasma haemominutum and *Candidatus* Mycoplasma turicensis. These species can cause anemia in cats and have a worldwide distribution.

Objectives; There was no previous information on hemotropic mycoplasma spp in cats in Iran and the Middle East. Accordingly we investigated the molecular presence, and clinical signs and hematological profile in cats infected with these microorganisms in Iranian cats.

Methods*;* Polymerase chain reaction (PCR) assays and cytology were performed on 100 blood samples collected from Iranian Shorthair cats. CBC and case history were also collected for each sample.

Results; By PCR, 22 (22%; 14-30%, 95% CI) samples were positive. The prevalence of *M. haemofelis, Ca.* M. haemominutum, and *Ca.* M. turicensis, was 63.63% (14/22), 54.54% (12/22) and 18.18% (4/22), respectively. Some double and triple co-infections were also found. Using the PCR as the reference method, cytology had poor sensitivity (27%) and reasonable specificity (89.74%). Male cats were at a higher risk of infection (P=.001). Cats older than 8 years were more frequently infected than the younger cats (P=.0018). Lower HCT (P=.018), RBC count (P=.028) and HGB concentration (P=.003) were also associated with hemoplasma PCR positive status.

Conclusions;,Based on this study, the most prevalent feline hemoplasma in *M. haemofelis,* and double and triple co-infections are also documented. Age and sex, as well as reduced RBC parameters,were predisposing factors for hemoplasma.

Introduction:

Feline hemoplasma organisms, previously known as *Hemobartonella* species, can cause hemolytic anemia.[1] Feline hemoplasmas comprise *Mycoplasma haemofelis, Candidatus* Mycoplasma haemominutum and *Candidatus* Mycoplasma turicensis.[2,3] Previously, cytology of blood smears was used to diagnose hemoplasma infection [4], but more recently, the

polymerase chain reaction(PCR) assay has become the method of choice for diagnosis due to its superior sensitivity and specificity.[4-6]

Co-infection with each of the 3 hemoplasmas with other pathogens such as *Bartonella spp*.and feline leukemia virus (FeLV) can result in outcomes different from each of these infections alone. A review of recent studies shows that these microorganisms may have a role in progression of retroviral, neoplastic, and immune-mediated diseases.[7, 8] However, it has also been demonstrated that hemoplasmas species has no effect on the severity or complications of some other pathogens.[9, 10]

There is currently little information available on the status of feline hemotropic mycoplasma infections in cats in theMiddle East, including Iran. Accordingly, the present study was conducted to investigate the prevalence, clinical signs and hematological profile associated with feline hemoplasma infection in blood samples of Iranian cats for the first time. In addition, molecular characterization was performed on positive samples.

2. Materials and Methods:

2.1. Sampling

Anticoagulated EDTA blood samples (FL Medical K3 EDTA K3E, Lot. F111332 2.5 ml tube, Torreglia, Italy) were collected from 50 male and 50 female Domestic Shorthair cats, which were presented to the small animal hospital of the College of Veterinary medicine, University of Tehran, for illness with clinical signs such as anorexia, lethargy, jaundice, diarrhea and vomiting, between August 2009 and April 2010. Historical data including background, previous diseases, elective surgeries, living with other cats, roaming or fighting were collected for each case.All samples were analyzed with a CBC and blood smears were made for

cytological examination (see below). The remainder of each sample was stored at -20◦ C for subsequent PCR analysis. The cats were divided into 3 groups, according to their age: <4 years, 4 - 8 years and > 8 years.

2.2. Hematological test

The CBC comprised RBC and WBC count, platelet count (PLT), HGB concentration, PCV, MCV, MCH, MCHC and RDW, and was performed with an automatic hemocytometer (Hema-screen 18, Hospitex diagnostic, Florence, Italy). The blood smears were stained with Giemsa for a differential blood cell count and detection of blood parasites on RBC (done by evaluating 20 fields oneach smear with an x100 objective). .

2.3. DNA extraction and polymerase chain reaction

DNA was extracted from 500 µl of EDTA blood using a commercially available kit (Fermentas#K0512, Burlington, Canada, 2010), according to the manufacturer's instructions. distilled water was used as a negative extraction control.

The PCR was performed on the extracted DNA with 4 different conventional PCR assays with related primers (Table 1). First, all 100 samples were screened for the presence of hemotropic mycoplasma species using universal primers. The positive samples were then subjected to 3 species-specific PCR tests to detect each of the 3 feline hemoplasma species (Table 4).

Briefly, 3 µl of the extracted DNA were added to a PCR master mix, including 14.35 µl of distilled water, 50 mM KCl, 200 µM of each dNTP, 1 µM of each primer, 1.5 mM of $MgCl_2$, 10 mM of Tris pH 8.3, and 2.5 units of Taq polymerase (all from Sinagen, Tehran, Iran). The actual PCR was performed with a final volume of 25 µl [11].

Positive controls for PCR amplification of specific sequences of *M. haemofelis, Ca*. M. haemominutum, and *Ca*. M. turicensis, were obtained from the School of Veterinary Sciences,

Bristol University, Bristol, UK and Bologna University, Bologna, Italy. These were DNA samples derived from cats infected with each of the three hemoplasma species. Distilled water was used as a negative PCR control for each PCR run,which comprised analysis of 7 unknown feline DNA samples.

The PCR was performed with the Techne /TC512 thermocycler, Chelmsford, England for the universal hemotropic mycoplasma PCR based on the PCR protocol published earlier.[11] The DNA samples yielding positive results with the universal PCR were then subjected to species-specific PCRs for *M. haemofelis* [12], *Ca*. M. haemominutum [12] and *Ca*. M. turicensis. [13]

A sample of 10 µl of the resulting PCR product and 1 µl of stain (Fermentas 6x, Burlington, Canada) were loaded onto a 1.5 % agarose gel (Sinagen) for electrophoresis. The electrophoresis chamber (Nojen PND 1000d, model Hu-95, Hu-150, Mashhad, Iran) was loaded with 0.5 x TBE buffer and run for one hour at 90 V. After electrophoresis, the gel was stained with ethidium bromide for 15 min and washed with deionized water for 5 min. The protein bands on the gel were evaluated with a UV transilluminator, TCP-20, Vilber, Eberhardzell, Germany.

2.4. Statistical analysis

Statistical analyses were performed using SPSS software, version 16.0 IBM, New York, United States. The normal distribution of data was evaluated by a 1-sample Kolmogorov-Smirnov test. Fisher's exact test and the independent T- tests were used for the analysis of data. The normally distributed data were expressed as mean ± standard deviation (SD) and a $P<.05$ was considered statistically significant.

3. Results

The results of the PCR analysis of all the samples are presented in Table 2 and Figures 1 to 4. Overall, 22 cats (22%) yielded positive PCR results with the hemotropic mycoplasma universal primers; the species specific PCRs on these samples yield the following results; 14 (14%) were positive for *M. haemofelis*, 12 (12%) were positive for *Ca*. M. haemominutum, and 4 (4%) were positive for *Ca*. M. turicensis. Thus the prevalence for the 3 species of hemoplasmas, *M. haemofelis* (Figure 2), *Ca*. M. haemominutum (Figure 3), and *Ca*. M. turicensis (Figure 4), was 63.63% (14/22), 54.54% (12/22) and 18.18% (4/22) respectively.Some of the cats were infected with more than one hemoplasma species (Table 2). The prevalence for the co-infection of *M. haemofelis* and *Ca*. M. haemominutum was 18.18% (4/22), whereas the prevalence for each of *M. haemofelis* and *Ca*. M. turicensis, *Ca*. M. haemominutum and *Ca*. M. turicensis, and triple infection, was 4.54 (1/22).

Of the 100 samples, 22 (22%, 95% CI) yielded positive result with the universal hemotropic mycoplasma PCR and 14 (14%; 95% CI) were positive on cytology; 8 of these cytology positive samples were negative by PCR. Using the PCR as the gold standard, cytology had a sensitivity of 27% and specificity of 89.74%.

The male cats were more at risk of hemoplasma infection (P=.001) compared to the female cats, with the former having an odds ratio of 20.4 times greater than the latter (95% CI; confidence level 6.33-66.1). The prevalence of hemoplasma infection in the cats older than 8 years was significantly (P=.0018) higher than that in those younger than 4 years, or between 4 to 8 years (Table 3).

CBCs showed that 10 out of the 22 hemoplasma-infected cats were classified as anemic, with a HCT<24%. Acomparison between the PCR-positive and PCR-negative cats (Table3) demonstrated that the PCR-positive cats had significantly lower HCTs (P=.018), RBC counts (P=.028) and HGB concentrations (P=.003). Total WBCs were significantly higher in the PCR-

positive cats (P=.021), accompanied by a left shift (P<.0001). Lymphocyte (P=.024) PLT counts (P=.008) and eosinophil counts (P=.004) were all lower in the PCR-positive cats (Table 4). In the peripheral blood smear of the cats, the presence of reactive lymphocytes, giant platelets, platelet aggregation, Howell jolly bodies, and, depending on the degree of anemia, anisocytosis and polychromasia, were observed.

The clinical signs of the PCR-positive cats based on history and clinical examination included anorexia, lethargy, jaundice, diarrhea, and vomiting in some cases. These clinical signs were most prominent in severely anemic cats. In contrast, some other infected cats showed no clinical signs (Table 2).

Some of the PCR-positive cats had a history of fighting or roaming, and had abscesses and open wounds. One animal (sample No. 11) with fever was suspected to be coinfected with another yet undiagnosed infectious pathogen. Another cat (sample No. 8) was diagnosed with concurrent kidney disease.

4. Discussion

This is the first study reporting the prevalence of feline hemoplasma species, together with associated hematology and epidemiological data,in cats in Iran. *M. haemofelis* was the most prevalent species, and clinical signs were more severe in cats coinfected with *Ca*. M. haemominutum and *Ca*. M. turicensis. In contrast, cats infected with *Ca*. M. turicensis alone or in combination with *Ca*. M. haemominutum appeared not anemic as specific clinical sign , indicating that the latter 2 species were not responsible for disease. Previous studies have described the prevalence of feline hemoplasmas in other geographic areas. According to most of these studies, *Ca*. M. haemominutum has the highest prevalence of the 3 species. For

instance, 17.3 % of an overall 18.9% of positive sampled cats in Italy, 13.4% infected cats of an overall 20.6% positive cats in Greece, and 15.3% in 17.1% infected cats in Australia[14-16], had *Ca. M. hemomintum,* which is in disagreement with our results showing *M. haemofelis* as the most prevalent species. However, a study on German cats described similar prevalence rates of feline hemoplasma species as in our study.[17] Overall, there is a paucity of data on co-infection of the hemoplasma species in other parts of the world. Nevertheless, co-infection with the 2 most common feline hemoplasma species *M. haemofelis* and *Ca*. M. haemominutum was reported in 3 cats in Brazil.[2]Dual and triple co-infections of *M. haemofelis* with the other 2species in latter study corroborates our findings.

A conventional PCR assay was used in the current study as this was the only PCR method available. Real-time quantitative PCR would have been useful to have enabled quantification of organism numbers in the blood of infected cats.[18]Stained smears, used previously as a diagnostic procedure in many laboratories, is not a sensitive diagnostic tool.[17] Our findings also indicate that the investigation of stained smears is not a very sensitive diagnostic method. A combination of conventional and real-time PCR assays was previously utilized to determine hemoplasma prevalence in cats in Italy.[14]Some other studies have applied real-time PCR to quantitatively determine hemoplasma organisms in cats, which could be of use in the diagnosis and monitoring of infection.[9, 19, 20] The present study was primarily aimed at describing the prevalence of infection with different hemoplasma species in Iranian cats, as opposed to describing infectious loads. Some studies describing feline hemoplasma infection prevalence have also reported co-infection with other potential anemia-inducing orgasnisms, such as piroplasmids (*Babesia* and *Theileria sp*.), FeLV and feline immunodeficiency virus (FIV) infections.[17, 21]Although no blood parasites such as *Babesia spp.* or *Theileria spp.* were identified during blood smear evaluation, unfortunately it was not possible to screen the cats in our study serologically or molecularly for such co-infections, which precluded us from

knowing whether co-infection might have contributed to the clinical signs or hematological abnormalities found.

similar to our study, an experimental investigation conducted on feline hemoplasma species, revealed that the infected cats were anemic having decreased hematologic parameters such as PCV, HGB and RBC counts .[22]Studies on naturally infected cats have alsoreported similar results as into our study.[9, 14] Our findings also indicated that age and sex were predisposing factors for feline hemoplasma infection insofar as the old and male cats in the present study were more positive than females. This may be due to the preference of older male cats to roam and fight with other cats.[9]

In a recent study in Switzerland, the morphological characterization of *Ca*. M. turicensis was determined as the latest known species of hemoplasma in cat.[23] The distribution and epidemiological aspects were evaluated in the present study; nonetheless, further studies are required to shed more light on this hemoplasma species.

In this study, we demonstrated for the first time the existence of feline hemoplasma infection in cats in Iran. Since the target population of this study consisted of sick cats further investigations, including healthy cats, and quantitative PCR studies are needed obtain more information on the different aspects of epidemiology, transmission and concurrent infection with other infectious agents such as FeLV in the general population of Iranian cats.

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Table 1. List of primers used to diagnose hemoplasma infection in the blood of sick Iranian cats

Species	**Name**	**Primer sequence**	**Size of PCR product (bp)**	**Reference**
1- Universal primers for hemotropic mycoplasma species	M2	5'-ATA-CGG-ATA-TTC-CTA-CG-3'	595-618	[11]
	M1	5'-TGC-TCC-ACC-ACT-TGT-TCA-3'		
2- *M. haemofelis*	M-hae-F	5'-TCG-AAC-GGA-YYT-TGG-TTT-CG-3'	1309	[12]
	M-hae-R	5'-CAA-ATG-AAT-GTA-TTT-TTA-AAT-GCC-CAC-3		

3- *Ca*. M. haemominutum	M-h1FB-F M-h2R-R	5’-AAG-TCG-AAC-GAA-GAG-GGT-TTA CTC-3’- 5’-TTW-AAT-ACG-GTT-TCA-ACT-AGT-ACT-TTC-TCC-3’	1354	[12]
4- *Ca*. M. turicensis	Mh1FA-F F2-R	5’-GAA-CTG-TCC-AAA-AGG-CAG-TTA-GC-3’ 5’-AGA-AGTTTC-ATT-CTT-GAC-ACA-ATT-GAA-3’	1317	[13]

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Table 2. Clinical signsand frequencyof the 3 feline hemoplasma species detected in 100 Iranian cats

Hemoplasma species detected	**Number of cats**	**Clinical signs**
M. haemofelis alone	8	Anemia (5), kidney disease (1), Roaming and Fighting background (7), lethargy (5), anorexia (6), Abscess & open wound (2), Jaundice (2), Vomiting & Diarrhea (1)
Ca. M. haemominutum alone	6	Abscess & open wound (1) Anorexia (5)
Ca. M. turicensis alone	1	No clinical signs reported
M. haemofelis & *Ca.* M. haemominutum	4	Anemia (4), pyrexia (1), Anorexia (3), Jaundice (3), lethargy (4), Roaming and

		Fighting background (4), Abscess & Open wound (2)
M. haemofelis & *Ca.* M. turicensis	1	Lethargy, Anorexia, Roaming and Fighting background
Ca. M. haemominutum & *Ca.* M. turicensis	1	Anorexia
All three hemoplasma species	1	Anemia, Jaundice, lethargy, Anorexia, Vomiting and Diarrhea

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Table 3. Comparison of the age and sex distribution in hemoplasma PCR-positive and PCR-negative cats.

Result	**Positive**		**Negative**		**Total**	
Age group (years)	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
<4	7	1	11	19	18	20
4-8	3	0	19	24	22	24
>8	8	3	2	3	10	6
Total	18	4	32	46	50	50

Table 4. Comparison of the CBC data in hemoplasma PCR-positive and PCR-negative cats

CBC data	Units	Positive cases (Mean±SD)	Negative cases (Mean±SD)	P value
RBC	(10^6/μL)	6.19 ± 2.48	7.50 ± 1.79	**0.028***
HCT	(%)	27.04 ± 11.90	33.80 ± 7.61	**0.018***
HGB	(g/dl)	10.07 ± 3.85	12.03 ± 2.47	**0.003***
MCV	(fl)	44.66 ± 10.70	46.43 ± 5.11	0.459
MCH	(pg)	16.78 ± 3.17	16.16 ± 2.44	0.401
MCHC	(g/dl)	36.66 ± 4.67	35.11 ± 3.12	0.155
WBC	(10^3/μL)	18.75 ± 12.67	11.97 ± 4.33	**0.021***

Segmented Neutrophils.	(10^3/μL)	11.76 ± 2.88	11.07 ± 1.83	0.187
Band Neutrophils.	(10^3/μL)	0.476 ± 0.189	0.188 ± 0.056	**<0.0001***
Lymphocytes	(10^3/μL)	2.90± 0.821	3.48 ± 1.08	**0.0244***
Monocytes	(10^3/μL)	0.21 ± 0.063	0.188± 0.048	0.731
Eosinophils	(10^3/μL)	0.30 ± 0.089	0.442± 0.167	**0.004***
Basophils	(10^3/μL)	0	0.0027 ± 0.023	0.641
platelets	(10^5/μL)	247.82 ± 139.21	340.92 ± 137.15	**0.008***

* P <.05

Figure1. PCR results with universal hemoplasma PCR primers on blood from sick cats in Iran. M: ladder 100 bp, C+: PCR positive control, 1-7: positive feline DNA samples, C-: PCR negative control. (198×300DPI)

Figure2. PCR results with specific primers for *Mycoplasma haemofelis*. M: ladder 100 bp, C+: positive Control, 1-10: positive feline DNA samples, C-: negative control. 201×151mm (300×300 DPI)

Figure3. PCR results with specific *Candidatus* Mycoplasma haemominutum primers. M: ladder 100 bp, C+: positive control, 1-3: positive feline DNA samples, C-: negative control. 201x151mm (300 x 300 DPI)

Figure4. PCR results with specific primers for *Candidatus* Mycoplasma turicensis. M: ladder 100 bp, C+: positive control, 1-3: positive feline DNA samples, C-: negative control. 151x201mm (300 x 300 DPI)